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In Vitro Production of Sexed Embryos for Gender Preselection: High-Speed Sorting of X-Chromosome-Bearing Sperm to Produce Pigs After Embryo Transfer

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ABSTRACT: The objectives for the present experiments were to apply sperm sexing technology to an in vitro production system with porcine oocytes obtained from slaughterhouse material. On six experimental days, ovaries were obtained from an abattoir, and cumulus-oocyte-complexes were matured in vitro. Semen was collected from mature boars of proven fertility and was sorted for X-chromosome-bearing sperm, using the Beltsville Sperm Sexing Technology incorporating the use of high-speed sorting. A total of 5,378 oocytes were submitted for in vitro fertilization (IVF). Of these, 559 ova were stained for cytogenetic analysis 18 h after IVF. From the remaining 4,819 ova, 1,595 cleaved, and 1,300 of the cleaved embryos were transferred into 26 synchronized recipients (5 control gilts for unsorted sperm, 21 gilts for X-sorted sperm). In a test of two fertilization media (FERT-A vs FERT-B) higher cleavage rates ($P < .05$) were obtained when FERT-B was used as a fertilization medium for unsorted (43.4 ± 5.1%) and sorted sperm (43.1 ± 1.1%;), whereas in FERT-A unsorted sperm gave a cleavage rate of 17.9 ± 4.4% and sorted sperm gave 30.4 ± 1.4%. Additionally, cleavage rates were higher ($P < .05$) after fertilization with sorted sperm vs unsorted sperm, independent of fertilization medium. Cytogenetic analysis of ova revealed that more oocytes with unsorted than with sorted sperm remained in Metaphase 2 arrest ($P < .05$). This was also independent of the fertilization medium. Monospermic fertilization rates were the same for IVF with unsorted or sorted sperm, independent of the fertilization system, except FERT-A with unsorted sperm ($P < .05$). Polyspermic fertilization rates were highest in FERT-B (37.6 ± 6.6). A total of 57 pigs were born from nine litters. Six litters from sexed sperm (X-sorted) produced 33 females (97%) and one male. Three litters from control transfers produced 23 pigs, 11 of which were female (48%). The sex ratio of the offspring was predicted based on the sort reanalysis of the sorted sperm for DNA content.

Key Words: Sex, Spermatozoa, Pigs, In Vitro, Fertilization, Embryo Transfer

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Introduction

Improving the reproductive efficiency of swine is dependent on the development of new biotechnologies that can be effectively applied to industry. Two such technologies are gender preselection and in vitro embryo production. The only proven sexing technology is the Beltsville Sperm Sexing Technology (Johnson et al., 1989; Johnson 1997). This method has consistently shown that offspring of the predicted sex can be produced when it is applied to rabbits (Johnson et al., 1989), swine (Johnson 1991; Rath et al., 1997; Abeydeera et al., 1998), and cattle (Cran et al., 1995, Seidel et al., 1997; Johnson et al., 1998). Recently, Johnson et al. (1998, 1999) demonstrated that sexed sperm production can be increased 10- to 15-fold using high-seed sperm sorting as opposed to standard-speed sperm sorting, and this increases the potential for wider use of the technology.

In vitro embryo production through in vitro fertilization (**IVF**) has been developed at several locations with various research groups (Nagai et al., 1984; Cheng, 1985; Yoshida, 1987; Rath, 1992; Day and Funahashi, 1996). Adaptation of IVF for use with sexed sperm was reported by Rath et al. (1997).

The objective for these experiments was to determine the effectiveness of using the sperm sexing technology in combination with in vitro maturation of ova and IVF and subsequent embryo transfer to produce pigs of predetermined sex. Additionally, the utility of two

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Table 1. Chemical components of the maturation, fertilization, and culture media (given as grams/liter if not indicated otherwise)

Constituent	TL-HEPES-PVA	MAT (NCSU 37)[a]	FERT-A	FERT-B	NCSU 23
NaCl	6.663	6.355	6.660	6.660	6.355
Na lactate 60% syrup, ml/L	1.868	—	1.850	1.850	—
Ca-Lactate·7.5 H_2O	—	—	—	1.690	—
$CaCl_2 \cdot 2H_2O$	.294	.250	.691	—	.250
$MgCl_2 \cdot 6H_2O$	.102	—	.100	.100	—
$MgSO_4 \cdot 7H_2O$	—	.293	—	—	.293
$NaHCO_3$	.168	2.106	2.100	2.100	2.106
KH_2PO_4	—	.162	—	—	.162
NaH_2PO_4	.041	—	.042	.055	—
KCl	.238	.356	.240	.240	.356
Na-Pyruvate	.022	—	.012	.012	—
Glucose	—	1.000	.900	.900	1.000
Glutamine	—	.146	—	—	.146
Sorbitol	2.186	2.186	—	—	—
Taurine	—	—	—	—	.875
Hypotaurine	—	—	—	—	.545
HEPES	2.383	—	—	—	—
Caffeine	—	—	.388	.388	—
Insulin	—	.005	—	—	—
PVA	.100	—	1.000	1.000	—
BSA	—	—	3.000	3.000	4.000
Gentamycin, μl/L	.500	—	—	—	.500
Penicillin G	.065	.065	—	—	.065
Streptomycin	—	.05	—	—	.05
Kanamycin sulfate	—	—	—	.100	—
Amikacin sulfate	—	—	.100	—	—

[a]On the day of use, 50 μmol mercaptoethanol, 1 mmol dbc-AMP, 10 μg/L IGF, .1 g/L L-cysteine, 10 IU/mL eCG, 10 IU/mL hCG, and 2% follicular fluid collected from small follicles are added.

different fertilization media was compared as part of the IVF procedure in order to increase fertilization rates and provide more transferable embryos. A preliminary report of these results has been given in abstract form (Long et al., 1998b).

Materials and Methods

In Vitro Oocyte Maturation

In six replicates, ovaries of pre- and peripuberal market gilts were collected at an abattoir and were transported to the laboratory within 5 h postmortem using procedures described earlier (Long et al., 1998a). Briefly, ovaries were washed with saline at room temperature and follicles (2 to 5 mm in diameter) were aspirated within 1 h after cleansing using an 18-gauge, short bevel needle, directly connected to a 50-mL polystyrene conical tube. Vacuum was set to .352 kg/cm^2, which was equivalent to a flow rate of 45-ml of H_2O per minute. Following follicular aspiration, pellets were allowed to settle for 7 min. The follicular fluid was discharged and cumulus oocyte complexes (**COC**) were washed twice with TL-HEPES-PVA medium (Table 1). Only oocytes with a complete and dense cumulus oophorus were matured in maturation medium (**MAT**) for 44 h in four-well dishes (50 COC per well; 500 μL) at 38.7°C in humidified air with 5% CO_2.The MAT was based on BSA-free NCSU-37 (Petters and Wells, 1993) supplemented with 5 mg/mL insulin, 10% follicular fluid (vol/vol), 50-μM β-mercaptoethanol, .6 mM L-cysteine, 10 ng/mL epidermal growth factor, .065 mg/mL penicillin G, and .05 mg/mL streptomycin. For the first 22 h of maturation, 10 IU/mL of eCG, 10 IU/mL of hCG (Intervet America, Millsboro, DE), and 1.0 mM db-cAMP (Sigma Chemical Co., St. Louis, MO) were added. Thereafter, COC were placed into fresh MAT without eCG, hCG, and db-cAMP and were cultured for another 22 h prior to fertilization.

Semen Preparation

The sperm-rich fractions of ejaculates were collected from mature boars of proven fertility on a regular collection schedule with the gloved hand method. For IVF with unsorted semen, 5 mL of the ejaculate was placed into a cooler (18°C) for 3 to 4 h while sorted sperm were being produced and were then washed three times in saline supplemented with 1% BSA prior to suspension in one of two fertilization media. A sample containing 150×10^6 sperm in 3 mL of Beltsville TS (BTS) and with Hoechst 33342 (Calbiochem, La Jolla, CA) from a 5 mg/mL stock solution, to give a final concentration of stain of 20.8 μM (Johnson, 1991). The samples were then incubated at 32°C for 45 min. After incubation of the sperm, 1.0 μL of FD&C#40 from a 25 mg/mL stock

solution was added and held at room temperature for 5 min. The FD&C#40 was effective in dampening the fluorescence of dead sperm, allowing them to be gated out of the sorting process (L. A. Johnson and G. R. Welch, unpublished data).

Flow Cytometric Sperm Sorting

A MoFlo high-speed cell sorter (Cytomation, Fort Collins, CO) modified especially for sorting sperm (Johnson and Pinkel, 1986; Johnson et al., 1999) was used to separate the X-chromosome- from Y-chromosome-bearing sperm. The machine parameters included an argon 5-W ion laser operating at 150 mW in the ultraviolet (UV, 351 to 364 nm multiline) as the excitation source. The modifications included a forward fluorescence detector (Johnson and Pinkel, 1986) and an orienting nozzle (Rens et al., 1998) replacing the beveled needle in order to orient a higher percentage of the sperm available for sorting into X and Y populations. The cell sorter sheath fluid was PBS with .1% BSA and .1% EDTA (wt/vol) at a pH of 7.2. Antibiotics were added to the sheath fluid (100 μg/mL penicillin G and 75 μg/mL streptomycin), and the fluid was sterile-filtered. Instrument sheath pressure was set at 2.81 kg/cm^2. The flow rate during sorting was approximately 8,000 sperm/s. This resulted in sort rates for each X and Y sperm population near 500 sperm/s (1.8×10^6). Sorted sperm were deflected into 15-mL polypropylene tubes that had been soaked with a 1% BSA solution prior to sorting and contained 50 μL of Test-Yolk (2%) buffer (Johnson et al., 1989, 1999). After the collection of 500,000 sperm per tube they were centrifuged at $350 \times g$ for 4 min and then resuspended in fertilization medium.

Small aliquots (50 μL, approximately 200,000 sperm) were sorted for flow cytometric sort reanalysis to determine the sort purities of the putative X and Y sperm populations (Johnson et al., 1987). Aliquots were sonicated (Branson Sonic Power, Danbury, CT) for several seconds and restained with 1.7 μL of Hoechst 33342 (5 mg/mL). Samples were then reanalyzed for sperm DNA composition. The resulting histograms were fitted to a double gaussian curve routine for the determination of X and Y sperm proportions. Reanalysis provided an evaluation of sort purity for prediction of the proportion of male and female pigs in the resulting offspring (Johnson et al., 1989).

In Vitro Fertilization

In vitro fertilization was performed in microdrops (50 to 100 μL, covered with light mineral oil, Sigma Chemical Co) using one of two fertilization media (FERT-A: Yoshida et al., 1993; Long et al., 1998a or FERT-B: Rath, 1996; Table 1). The FERT-A contained .691 g/L $CaCl_2$, whereas FERT-B was supplemented with 1.690 g/L Ca-lactate. Additionally, the content of NaH_2PO_4 was 24% higher in FERT-B (.042 vs .055 g/L). Prior to fertilization matured oocytes with at least three expanded cumulus cell layers were denuded, and 35 to 55 oocytes were placed into each microdrop together with 75 unsorted sperm/oocyte or 35 sorted sperm/oocyte. These sperm concentrations were determined in previous experiments (our unpublished observation) to give lowest rates of polyspermy without diminishing the in vitro fertilization rates. After 6 h, fertilized oocytes were transferred into four-well tissue culture plates (Nunc, Roskilde, Denmark) filled with 500 μL of culture medium NCSU 23 (Petters and Reed, 1991; Table 1) and were cultured for 42 to 44 h.

Due to the experimental design, cytogenetic tests for the degree of in vitro maturation had to be combined with the analysis of mono- and polyspermic fertilization, allowing some detailed information on maturation to be lost. Randomly selected subsets of zygotes were removed from the culture plates 18 h after onset of fertilization and were prepared for morphological assessment (Long et al., 1998a). Briefly, embryos were fixed in 2% paraformaldehyde in PBS with 10% Triton X-100 for 1 h at 37°C and were stained with Hoechst 33342 (2 μg/mL Hoechst 33342 in PBS with 20% glycerol and 100 mg/mL of 1,4 diazabicyclo [2.2.2] octane [Sigma Chemical Co.]). Evaluation was performed under a Zeiss Axiovert-135 microscope (Zeiss, Thornwood, NY) using a 40× Hoffmann-Zeiss objective (epifluorescence illumination 100-W mercury bulb, UV filter [#487901; 365 nm]).

Embryo Transfer

Mature crossbred gilts were used as recipients. They were fed 5 mL of Regumate (20 mg Altrenogest; A. J. Buck, Baltimore, MD) per day over 14 d. Ovulation was induced with 500 IU of hCG, and the cycle of recipients was delayed 24 h (asynchronous) to what would be expected from the developmental stage of the IVF embryos. Approximately 44 to 48 h after IVF, cleaved embryos (2 to 8 cell stages) were washed in 2 mL of NCSU 23 supplemented with 2.38 g/L HEPES. Modified plastic straws (Rath et al., 1994) were used as embryo carrier and transfer instruments. Up to 25 embryos were placed into each plastic straw. Embryos were transferred into each oviduct as described earlier (Rath et al., 1997). Briefly, gilts were anesthetized, and the abdomen was opened with midventral incision. Ovaries and oviduct were exposed, and functional structures were counted on each ovary. The plastic straw was introduced as deeply as possible through the ovarian fimbria into the oviductal ampulla, and embryos were pushed into the lumen when a metal mandrin was inserted into the cotton-plugged end of the plastic straw. Any mature (≥ 5 mm) but nonovulatory follicles were punctured with a 23-gauge needle, in order to induce luteinization. Following surgery, recipients were checked daily for estrus using a teaser boar.

Data were analyzed with the "Sigma Stat" program (Jandel Corporation, San Rafael, CA). All data were tested for normal distribution with the Kolomogorov-

Table 2. Cleavage rates after in vitro fertilization with sorted and unsorted sperm using fertilization media FERT-A and FERT-B

	Unsorted sperm		Sorted sperm	
Item	Fert-A	Fert-B	Fert-A	Fert-B
Replicates[a]	13	13	44	32
Oocytes to be fertilized	503	436	2,366	1,514
Cleaved	74	177	691	653
Cleavage rate, %, mean ± SEM	17.9[b] ± 4.4	43.4[c] ± 5.1	30.4[d] ± 1.4	43.1[c] ± 1.1

[a]Each microdrop was considered as one replicate.
[b,c,d]Two way ANOVA: means with different superscripts differ ($P < .05$).

Smirnov test. Additionally data were tested for equal variance. If necessary, data were transformed using arcsine transformation. Statistical differences were tested with a chi^2 test, or Kruskal-Wallis ANOVA on ranks (performing all multiple comparison procedures with Dunn's method), or with a two-way ANOVA using a GLM procedure. In these cases, the multiple comparison procedure was performed with the Bonferroni *t*-test. Data are expressed as means ± SEM. A probability value of $P < .05$ was considered to be significant.

Results

Average motility of the raw semen used in the study was 81.4 ± 3.4% (means ± SEM), and acrosomal integrity was 93.6 ± 1.4%. Sperm motility following sorting was 60% in all cases. Sort purity, as determined from aliquots sorted in sequence with the sperm sorted for use in IVF, was 97 ± .4% for X-bearing sperm based on reanalysis of sorted sperm for DNA content. After in vitro maturation, 5,378 cumulus oocyte complexes were fertilized in vitro, and 559 embryos were stained 6 h after fertilization. From the remaining 4,819 fertilized oocytes, 1,595 embryos (33.0%) cleaved.

Cleavage rates were analyzed with a two-way ANOVA. Allowing for effects of differences in semen treatment, media effects were significantly different (LSM ± SEM: FERT-A 28.6 ± 3.5; FERT-B 67.5 ± 3.6; P < .001). Allowing for effects of differences in IVF media, mean values of unsorted and sorted semen were significantly different (LSM ± SEM: unsorted 39.5 ± 4.3; sorted 56.6 ± 2.6). No significant interactions between media and semen treatment were present ($P = .210$). The multiple comparison showed that, in general, cleavage rates were lower using FERT-A as IVF medium compared with FERT-B ($P < .05$). Additionally, significant differences were found between unsorted and sorted semen when FERT-A was used as IVF medium, whereas this was not the case using FERT-B (Table 2).

Cytogenetic data obtained from fluorescence staining 18 h after IVF (Table 3) were analyzed for percentages of unfertilized Metaphase 2 stages and monospermic (2 pronuclei) and polyspermic (> 2 pronuclei) fertilization with two-way ANOVA. For oocytes remaining in Metaphase 2 stage that potentially could have been fertilized, main effects of FERT-A and FERT-B were different (LSM ± SEM: FERT-A 47.8 ± 3.7; FERT-B 29.1 ± 3.8; $P < .001$), allowing for effects of differences in sperm treatment. Allowing for effects of differences in media, sperm treatment also had significant main effects (LSM ± SEM: unsorted 29.3 ± 4.4; sorted 27.5 ± 2.9; $P < .001$). However, no interaction between the variables was found ($P < .425$). Most matured oocytes remained unfertilized (means ± SEM, 66.1 ± 5.8%) using FERT-A with unsorted semen ($P < .05$) compared to the other IVF groups. The lowest percentage of oocytes remaining unfertilized was found when sorted sperm were used for IVF in FERT-B (21.3 ± 4.8%).

The percentage of zygotes that had two pronuceli was lowest in FERT-A after IVF with unsorted semen. No other treatment groups differed significantly. Main effects remain questionable but were not significant for the kind of medium used, allowing for effects of differences in sperm treatment (LSM ± SEM: FERT-A: 19.4 ± 2.5; FERT-B: 26.7 ± 5.6; $P < .051$). Allowing for effects of difference in media, semen treatment affected monospermic fertilization rates (LSM ± SEM: unsorted 18.9 ± 3.0; sorted 27.2 ± 2.0; $P < .026$). An interaction between the main effects cannot be excluded ($P < .053$). Polyspermic fertilization was lower with unsorted than with sorted sperm. The highest rates of polyspermy were found when sorted sperm were used for fertilization in FERT-B. The differences of main effects of media were significant, allowing for effects of differences in semen treatment (LSM ± SEM: FERT-A 12.1 ± 4.0; FERT-B 28.9 ± 4.1; $P < .006$). Allowing for effects of different media, semen treatment had a significant effect on polyspermy (LSM ± SEM: unsorted 10.9 ± 4.8; sorted 30.1 ± 3.2; $P < .002$). A significant interaction was not found ($P = .782$).

Embryo transfer was performed in five recipients using 250 control embryos that were produced with unsorted sperm. Another 1,050 embryos produced with sorted sperm were transferred into the oviductal ampullae of 21 recipients; this is equivalent to a total of 50 embryos per recipient. Embryos were deposited in groups of 25 into each uterus horn. After IVF with unsorted semen, three animals (60%) became pregnant, and 23 pigs were born. Fifty-two percent were male. Average litter size was 7.7 ± 2.3 (means ± SEM), ranging

Table 3. Staining results of porcine ova 18 h after onset of in vitro fertilization (IVF)

	Unsorted		Sorted	
Stage	FERT-A, %	FERT-B, %	FERT-A, %	FERT-B, %
	Mean ± SEM			
Metaphase 2	66.1[a] ± 5.8	37.8[b] ± 3.1	37.1[b] ± 4.3	21.3[c] ± 4.8
2 Pronuclei	11.7[a] ± 4.1	22.8[b] ± 3.6	26.4[b] ± 2.8	27.9[b] ± 3.1
> 2 Pronuclei	1.7[a] ± 1.1	19.5[ab] ± 4.2	22.5[b] ± 13.3	37.6[c] ± 6.6

[a,b,c]Means within a row with different superscripts differ significantly ($P < .05$). Two-way ANOVA, general linear model.

from 4 to 12 pig per litter. When semen sorted for X-chromosome-bearing sperm was used for IVF, six pregnancies (28.6%) were obtained, and 34 pigs were born. Ninety-seven percent were female. The average litter size was 5.8 ± .8, ranging from 4 to 8 pigs per litter. Comparison of these data showed that the number of farrowings per transfer were not different (chi^2 test: $P = .396$). Sorting for X-chromosome-bearing sperm changed the ratio of female offspring born ($P < .05$). The litter size after IVF with sorted semen was less ($P < .05$) than with transfer of embryos derived from IVF with unsorted semen (Table 4). When relating the total number of pigs born to the number of embryos transferred, more pigs were born when they were produced with unsorted semen compared with IVF with sorted semen (9.2 vs 3.2%; chi^2 test: $P < .001$). However, when comparing the number of female pigs born after IVF with either one of the sperm categories, there were no differences (4.4 vs 3.2%; chi^2 test: $P = .449$).

Discussion

The main goal for this experiment was to produce female pigs using sperm bearing an X-chromosome for in vitro fertilization of in vitro matured oocytes. In a previous embryo transfer experiment, we showed that pigs can be produced by such a technique with in vivo matured oocytes (Rath et al., 1997). Additionally, Abeydeera et al. (1998) and Long et al. (1998b) showed that pigs can be derived from in vitro maturation (**IVM**) and subsequent IVF with sorted semen using in vitro matured oocytes. Although satisfactory numbers of sexed sperm were obtained for IVF in previous studies using the standard sorting system (Johnson, 1991; Rath et al., 1997), the newest sperm sorting technology available was used in the present study. This system, which uses a high-speed cell sorter in combination with a new orienting nozzle (Johnson et al., 1998, Rens et al., 1998) improves the output of sorted sperm approximately 10 to 15 times compared with the original Epics V modified cell sorter on which the Beltsville Sperm Sexing Technology was developed.

Forty-eight hours after IVF, embryos were transferred surgically into the oviduct of synchronized recipients. As predicted by reanalysis of the sorted semen, 97% of the offspring in this particular group were female; one pig was male. The sex of the offspring in the control group with unsorted semen was 52% male and 48% female (12 and 11, respectively). The difference in the number of farrowings was not significant but indicates that the developmental capacity of IVM/IVF embryos is still limited and is numerically further reduced when sorted semen is used for fertilization. The litter size was less for sows that farrowed pigs from sorted sperm rather than unsorted sperm. This fact needs further investigation to determine whether the reason for the lower litter size might be a higher proportion of embryonic death associated with sorted sperm, which is consistent with previous results using sorted sperm (Johnson, 1991). Although the litter size was smaller when sorted sperm was used for IVF and only 3.2% of the potential oocytes fertilized with sorted sperm, produced offspring vs 9.2% using unsorted sperm, the total number of female pigs did not differ

Table 4. Pregnancy and farrowing results after transfer of embryos produced from in vitro maturation and in vitro fertilization (IVF) with sorted and unsorted sperm

Sperm used	No. of transfers/ farrowings	Pigs born	No. male	No. female	% Female	Litter size (means ± SEM) (min–max)
Unsorted	5/3[a]	23	12[b]	11[b]	48	7.7[d] ± 2.3 (4–12)
Sorted for X	21/6[a]	34	1[b]	33[c]	97	5.8[e] ± .8 (4–8)

[a]Chi^2 test: not significant ($P = .396$).
[b,c]Kruskal-Wallis ANOVA on ranks: comparison of male and female pigs within a column $P < .05$.
[d,e]Kruskal-Wallis ANOVA on ranks: comparison of litter sizes $P < .05$.

between the groups. In the future, this ratio has to be improved to gain more benefits from sorting on the number of produced offspring of a certain sex.

In this experiment, 50 embryos were transferred into each recipient, almost double the number usually applied in normal embryo transfer protocols (Niemann and Meinecke, 1993). The purpose of this approach was to increase the chances of the sow carrying a litter of at least four pigs. Further research will focus on specific evaluation characteristics of IVF embryos prior to embryo transfer to predict their developmental capacity and allowing the transfer of fewer embryos per recipient.

The second purpose of these experiments was to test two IVF media in order to use as many in vitro matured oocytes as possible to produce transferable embryos. A total of 5,378 in vitro matured oocytes were fertilized in either one of the IVF media with sorted and unsorted semen. Cytogenetic analysis of ova showed that more potentially matured oocytes remained unfertilized (Metaphase 2) when FERT-A was used for IVF with unsorted sperm. Fewer oocytes remained unfertilized using FERT-B and sorted semen. This depends on sperm treatment effects and confirms our previous experiences fertilizing in vivo matured oocytes with sorted and unsorted sperm (Rath et al., 1997). Because the source of oocytes and semen was identical between groups, the reason for these differences is not clear. However, it might be due to the ratio of oocytes and sperm cells used in this particular IVF system. We tried to optimize this ratio before we started the experiment and used sperm numbers that were found best in the previous studies (Rath et al., 1997; Long et al., 1998a). But in context with the rate of mono- and polyspermic fertilization, the number of unsorted sperm might have been too low for IVF in FERT-A. Higher sperm concentration used with FERT-B gave better fertilization, but polyspermic fertilization increased with higher fertilization rates and was maximal when FERT-B was used for IVF with sorted sperm. Major reasons for polyspermic fertilization under in vitro conditions may be the presence of too many sperm in relation to the number of oocytes, insufficient ability of the oocytes to release cortical granules (Cran and Cheng, 1986), and incompetent cumulus cells. Additionally, sperm cells are not preselected for fertilization in vitro, compared to their selective passage through the uterotubal junction under in vivo conditions, although polyspermic fertilization has also been observed in pigs under in vivo conditions to a certain extent (Hunter, 1976). Additionally, factors in the follicular fluid that is added to the maturation medium may promote polyspermy (Yoshida et al., 1992). In vivo, the ratio of sperm per oocyte is regulated partially by the endocrinologically controlled release of sperm from the uterotubal junction (Hunter, 1995). In addition, the expanding, mucified cumulus oocyte complex may produce signals that regulate sperm release from the uterotubal junction to the isthmus (Hunter, 1996), which is impossible in vitro. It has also been suggested that competent sperm may swim up to the higher temperature gradient that exists in the postovulatory ampulla-isthmic junction (Hunter, 1998), a point that has to be considered for future IVF models. Currently, the only way to limit polyspermic fertilization is to reduce the number of sperm per oocyte without a significant reduction of the monospermic fertilization rate (Rath, 1992; Coy et al., 1993). In the present experiment, an optimal sperm concentration was not applied. Consequently, no beneficial effect was detected for either one of the fertilization systems in terms of maximal fertilization rates.

Cleavages rates were affected by fertilization media and semen treatment. The FERT-B was superior to FERT-A, independent of the semen treatment. However, as discussed before, the number of sperm per oocyte might have been suboptimal for usage with unsorted sperm. Because sorted sperm, in general, fertilized better than unsorted sperm, effects may be due to an advanced change in sperm membranes that occurs through the sorting process in a kind of artificial precapacitation (Maxwell and Johnson, 1997). The effect on sperm membranes seems to be related to the pressure changes associated with sorting. Thus, sorted sperm do not need to undergo an additional maturation period for capacitation, as is required for unsorted sperm after semen collection. Unsorted sperm were capacitated in this experiment by storage at room temperature for 3 h (Nagai et al., 1984), which is sufficient for IVF (Rath, 1996), but this was never directly compared to the mechanical capacitation procedures used in the cell sorter. Our experiment was not designed to elucidate the effects of FERT-B and FERT-A on sperm membrane changes, but the availability of calcium ions in combination with an increased phosphate concentration in FERT-B might have been responsible for higher cleavage rates. Calcium ions are directly involved in the capacitation process (Singh et al., 1978; Yanagimachi, 1982; Irvine and Aitken, 1986; Stock and Fraser, 1989). As we have shown earlier (Laurincik et al., 1995), an asynchronous development of both pronuclei that can be caused by delayed capacitation and acrosome reaction reduces cleavage rates but not fertilization rates, just as we have seen in this experiment.

Implications

The application of sex preselection to swine production worldwide would have a dramatic impact on breeding schemes, facility design, market strategies, and reproductive efficiency. The Beltsville Sperm Sexing Technology applied to boar sperm is capable of producing sexed sperm for use with in vitro fertilization (IVF) and embryo transfer to produce offspring of the desired sex. Our data indicate that if one uses pig sperm sorted for the X-chromosome for IVF 97% of the offspring will be female. Implementation of these procedures could lead to greater efficiency in producing swine for particular markets while at the same time advancing genetic

improvement at a faster rate. These studies also demonstrate that the production of sorted sperm using high-speed technology (5×10^6) is still insufficient to use sexed semen for conventional artificial insemination of swine.

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